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Expression, purification, crystallization and X-ray analysis of 3-quinuclidinone reductase from *Agrobacterium tumefaciens*

(*R*)-3-Quinuclidinol is a useful chiral building block for the synthesis of various pharmaceuticals and can be produced from 3-quinuclidinone by asymmetric reduction. A novel 3-quinuclidinone reductase from *Agrobacterium tumefaciens* (AtQR) catalyzes the stereospecific reduction of 3-quinuclidinone to (*R*)-3-quinuclidinol with NADH as a cofactor. Recombinant AtQR was overexpressed in *Escherichia coli*, purified and crystallized with NADH using the sitting-drop vapour-diffusion method at 293 K. Crystals were obtained using a reservoir solution containing PEG 3350 as a precipitant. X-ray diffraction data were collected to 1.72 Å resolution on beamline BL-5A at the Photon Factory. The crystal belonged to space group *P*2₁, with unit-cell parameters *a* = 62.0, *b* = 126.4, *c* = 62.0 Å, β = 110.5°, and was suggested to contain four molecules in the asymmetric unit (*V*_M = 2.08 Å³ Da⁻¹).

1. Introduction

In the chemical, food and pharmaceutical industries, chiral compounds are indispensable building blocks for the production of chemical catalysts, liquid crystals, flavours, agrochemicals and drugs (Dausmann *et al.*, 2006). In chemical processes, reaction conditions such as high temperature or high pressure or additives such as acids or alkalis are required to accelerate the reaction, while the production of unstable compounds requires milder reaction conditions. Enzymatic reactions can provide mild conditions since they achieve catalysis in a neutral solution. Additionally, enzymatic asymmetric reduction shows remarkable chemoselectivity, regioselectivity and stereoselectivity (Wandrey, 2004), which makes enzymes more efficient for the production of chiral compounds.

(*R*)-3-Quinuclidinol is a chiral building block that is used in the synthesis of various pharmaceuticals such as talsacidine and revatropate (Rzeszutarski *et al.*, 1988; Bietti *et al.*, 1990; Cross & Stobie, 1993; Takeuchi *et al.*, 1996; Alabaster, 1997; Ward *et al.*, 1998; Leusch *et al.*, 2000; Ishihara *et al.*, 2004). This chiral compound is synthesized using several chemical reactions, whereas it can easily be synthesized by the stereospecific reduction of 3-quinuclidinone by the enzyme 3-quinuclidinone reductase. This enzyme was first isolated from *Rhodotorula rubra* (RrQR) and required NADPH as a cofactor (Uzura *et al.*, 2009; Takeshita *et al.*, 2009). A novel 3-quinuclidinone reductase was recently isolated from *Agrobacterium tumefaciens* (AtQR) which also stereospecifically produces the (*R*)-enantiomer of 3-quinuclidinol. Based on amino-acid sequence identity (43% identity to RrQR), AtQR was concluded (like RrQR) to belong to the short-chain dehydrogenase/reductase family, the members of which contain a Rossmann fold for binding to NAD(P)H. However, AtQR requires NADH as a cofactor rather than NADPH. In addition, AtQR shows a 100-fold higher substrate affinity than RrQR (unpublished data), which make it more efficient for 3-quinuclidinol production.

In order to elucidate the structural basis of its high substrate affinity and cofactor specificity, we are currently undertaking structural analysis of AtQR. Here, we report the expression, purification, crystallization and X-ray diffraction analysis of this enzyme.

2. Materials and methods

2.1. Expression and protein purification

An AtQR-coding sequence (accession No. AB469148) was inserted into the pET-28a(+) vector (Novagen) between the *Nde*I and *Bam*HI sites for the production of a recombinant protein with an N-terminal His₆-tag sequence (MGSSHHHHHHSSGLVPRGSH). The protein was overexpressed using *Escherichia coli* Rosetta (DE3) (Novagen) cells. Expression of the AtQR gene was induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 298 K when the optical density at 600 nm reached 0.6. After overnight culturing, the cells were harvested by centrifugation at 5180g for 10 min and were frozen at 193 K.

Figure 1
Typical crystals of AtQR grown at 293 K using PEG 3350 as precipitant.

The frozen cells were resuspended in lysis buffer consisting of 20 mM Tris–HCl pH 7.4, 500 mM NaCl, 30 mM imidazole, 1 mM dithiothreitol and were disrupted by sonication. After centrifugation at 40 000g for 30 min, the supernatant was applied onto an Ni–NTA Superflow column (Qiagen). After washing with lysis buffer, the fusion protein was cleaved on the column using thrombin protease (GE Healthcare) overnight at 277 K. Three additional amino acids (GSH) remained at the N-terminus of the AtQR-coding sequence. The cleaved protein was eluted with lysis buffer and dialyzed against 20 mM Tris–HCl pH 8.0. The dialyzed solution was loaded onto a DEAE Sepharose column (GE Healthcare) and the protein was eluted with buffer containing 200–500 mM NaCl. The fraction containing AtQR was concentrated and applied onto a HiLoad Superdex 200 prep-grade column (GE Healthcare) equilibrated with buffer consisting of 20 mM Tris–HCl pH 8.0, 200 mM NaCl. The purified protein solution was dialyzed against 20 mM Tris–HCl pH 7.0 and concentrated to 15 mg ml^{−1}. The protein concentration was determined from the absorbance at 280 nm using a molar extinction coefficient of 17 992 M^{−1} cm^{−1} (Pace *et al.*, 1995) and a molecular mass of 27 561 Da.

2.2. Crystallization and X-ray diffraction data collection

For the crystallization of AtQR with its cofactor, 2 mM NADH was added to the protein solution. Initial crystallization trials were performed by the sitting-drop vapour-diffusion method in 96-well plates (Corning) using the sparse-matrix screening kits Crystal Screen HT and Index HT (Hampton Research) at 293 K. Drops containing equal volumes (0.5 μ l) of protein solution and crystallization solution were equilibrated against 40 μ l reservoir solution. For refinement of the crystallization conditions, protein solution and reservoir solution were mixed in equal volumes (1 μ l) and the drops were equilibrated

Figure 2
An X-ray diffraction image (0.3° oscillation) from an AtQR crystal. The data set was analyzed at 1.72 Å resolution (indicated by the circle).

Table 1

Crystal parameters and data-collection statistics for AtQR.

Values in parentheses are for the highest resolution shell.

Beamline	BL-5A, PF
Wavelength (Å)	1.0000
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 62.0$, $b = 126.4$, $c = 62.0$, $\beta = 110.5$
Resolution range (Å)	20.0–1.72 (1.76–1.72)
Observed reflections	332879 (10121)
Unique reflections	92664 (5628)
Multiplicity	3.6 (1.8)
Completeness (%)	97.9 (80.7)
$R_{\text{merge}}^{\dagger}$ (%)	4.3 (16.0)
$\langle I/\sigma(I) \rangle$	21.7 (5.1)
Wilson B factor (Å ²)	14.9

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th intensity measurement of reflection hkl , including symmetry-related reflections, and $\langle I(hkl) \rangle$ is its average.

against 0.5 ml reservoir solution at 293 K in 24-well plates (Hampton Research) using the sitting-drop vapour-diffusion method.

A single crystal of AtQR was picked up in a nylon loop (Hampton Research) and transferred to reservoir solution containing 20% (v/v) ethylene glycol. The crystal was mounted for flash-cooling at 100 K using a nitrogen stream. X-ray diffraction data were collected on beamline BL-5A at the Photon Factory (PF), Tsukuba, Japan using an ADSC Quantum 315r CCD detector. The distance between the crystal and detector was set to 249.3 mm. A data set was composed of 600 images and collected using a 0.3° oscillation. The diffraction data were indexed, integrated and scaled using *XDS* (Kabsch, 1993).

3. Results and discussion

We have established methods for the preparation and crystallization of AtQR. After the crystallization conditions had been refined, crystals suitable for X-ray analysis were obtained using a reservoir solution consisting of 0.2 M ammonium acetate, 0.1 M HEPES pH 8.5, 24% (w/v) PEG 3350 (Fig. 1). The X-ray diffraction data were obtained from a rod-shaped crystal with approximate dimensions of 50 × 50 × 300 µm. The crystal belonged to the primitive monoclinic space group $P2_1$, with unit-cell parameters $a = 62.0$, $b = 126.4$, $c = 62.0$ Å, $\beta = 110.5^\circ$. Fig. 2 shows an X-ray diffraction image from an AtQR crystal. X-ray diffraction data to 1.72 Å resolution were used for structure analysis, since a data completeness of 80% or better would be required for good quality. The data-collection statistics are listed in Table 1. The Matthews coefficient and solvent content of the native crystal were estimated to be 2.08 Å³ Da^{−1} and 41.03%, respectively (Matthews, 1968), suggesting that the crystal contained four molecules of AtQR in the asymmetric unit. We chose *meso*-2,3-butanediol dehydrogenase from *Klebsiella pneumonia* (PDB entry

1geg; Otagiri *et al.*, 2001) as a molecular-replacement search model because it also belongs to the SDR family and its sequence is the most similar to AtQR (43% identity) among proteins for which structures are available. Molecular replacement was carried out using *Phaser* (McCoy *et al.*, 2007). A solution was found with a translation-function Z -score (TFZ) of 23.1 and a log-likelihood gain (LLG) of 828.8 for four protomers. The four protomers in the asymmetric unit formed a tetramer.

The high-resolution data should help us to more accurately analyze the structural basis of the high substrate affinity and cofactor specificity. For further study, mutation analysis based on the crystal structure and structural analysis of the substrate-bound protein will be carried out.

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